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<b>(21) International Application Number:</b> PCT/EP92/02531 <b>(22) International Filing Date:</b> 4 November 1992 (04.11.92)  <b>(30) Priority data:</b> 791,933 5 November 1991 (05.11.91) US  <b>(71) Applicant (for AT only):</b> SANDOZ-ERFINDUNGEN VERWALTUNGSGESELLSCHAFT M.B.H. [AT/AT]; Brunner Strasse 59, A-1230 Vienna (AT).  <b>(71) Applicant (for DE only):</b> SANDOZ-PATENT-GMBH [DE/ DE]; Humboldtstrasse 3, D-7850 Lörrach (DE).  <b>(71) Applicant (for all designated States except AT DE):</b> SAN- DOZ LTD. [CH/CH]; Lichtstrasse 35, CH-4002 Basle (CH).		<b>(72) Inventors:</b> NICHOLS, Scott, E. ; 499 Merlot Drive, Fre- mont, CA 94539 (US). PAULY, Michael, H. ; 1917 Sherman, Boise, ID 83702 (US). SINIBALDI, Ralph, M. ; 1780 Acacia Court, Fremont, CA 94536 (US). WEEKS, Donald, P. ; 6340 Rainier Drive, Lincoln, NB 68510 (US). BAKER, Frederick, Charles ; Baselmattweg 159, CH-4123 Allschwil (CH). DUNCAN, Marian, L. ; 715 Everett Street 4, Palo Alto, CA 94301 (US).  <b>(74) Common Representative:</b> SANDOZ LTD.; Patents and Trademarks Div., Lichtstrasse 35, CH-4002 Basle (CH).  <b>(81) Designated States:</b> AU, HU, JP, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> IMPROVED SUPERSWEET CORN  <b>(57) Abstract</b>  Improved supersweet corn plants, in addition to their naturally occurring homozygous recessive sh-2 or bt-2 genes, also have an ADP-GPP subunit gene under the control of a heterologous promoter. The heterologous promoter may either be developmentally delayed so that it is active only after approximately 25 or 30 days post pollination, or it may be an inducible promoter. The improved kernels are supersweet when harvested for food, but are starchy when harvested as a seed crop. Alternatively an anti-sense ADP-GPP subunit gene under the control of an inducible promoter or a promoter which is active during early endosperm development may be used to transform a sweet corn plant such that transcription of the anti-sense gene blocks ADP-GPP synthesis giving rise to supersweet kernels.		

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## IMPROVED SUPERSWEET CORN

## FIELD OF THE INVENTION

This invention relates to plant biotechnology and specifically to corn, alternatively known as maize, Zea mays or Indian corn. In particular the invention relates to supersweet corn with improved qualities, methods of producing the improved supersweet corn, vectors for the genetic engineering of the corn, DNA used to transform corn, and genomic clones containing the desired DNA.

## BACKGROUND OF THE INVENTION

The endosperm is a major site of starch deposition during maize kernel development. Many genetic loci affecting carbohydrate metabolism are known and some have been biochemically characterized. When some of these genes are not expressed, the result is generally a decrease in starch biosynthesis and a concomitant accumulation of sucrose to give a seed product commonly referred to as sweet corn and used as a human food product. Corn in which the levels of starch biosynthesis are normal is commonly referred to as field corn, and is often used for animal feed.

Some of the genes involved in sweet corn production are Su (sugary-1), Sh-2 (shrunken-2) and Bt-2 (brittle-2). Sweet corn results from the presence of a homozygous recessive allele at the sugary-1 locus on chromosome 4, designated su. When su is present, there is a 3-5 fold increase in the percentage of sucrose (dry weight) relative to wild-type Su lines. The Sh-2 and Bt-2 genes, both involved in starch production, encode subunits of the enzyme ADP-glucose pyrophosphorylase (ADP-GPP)

( $\alpha$ -D-glucose-1-phosphate adenyl transferase, EC 2.7.7.27).

ADP-GPP catalyzes the reversible synthesis of ADP-glucose and pyrophosphate from ATP and glucose-1-phosphate. The equilibrium constant for this reaction is unity and the reaction is driven by pyrophosphate hydrolysis. ADP-glucose is the glucosyl donor for starch chain elongation catalysed by starch synthetase. ADP-GPP is made up of four subunits: two subunits of a 60 kDa polypeptide encoded by the Sh-2 gene (hereinafter referred to as the Sh-2 protein), and two subunits of a 55 kDa polypeptide encoded by the Bt-2 gene (hereinafter referred to as the Bt-2 protein). When either the Sh-2 or Bt-2 gene is present in the homozygous recessive condition (designated sh-2 or bt-2 respectively), the 60 kDa or the 55 kDa subunits (respectively) are not properly synthesized, and little or no functional ADP-GPP is made. As a result, starch synthesis is impaired, sucrose accumulates to 2-4 times the levels of normal sweet corn, and the corn is "supersweet".

While supersweet corn is becoming quite popular among consumers, the supersweet corn plants have many agronomic disadvantages. The dry weight of the kernel is reduced relative to Sh-2 lines due to the decrease in starch synthesis. Also, the accumulation of endosperm zein proteins in supersweet lines is only approximately half that of Sh-2 lines. As a result of these factors, dry-down and germination are adversely affected. While germination rates in field corn approach 100%, those of supersweet lines can be as low as 10% and tend to be approximately 50%.

While dry field corn seeds are plump or round with a small indentation at the crown of the kernel (so-called dent corn), dry supersweet seed is shrunken and collapsed in its appearance, and is rather fragile. It has many angular faces and indentations which may result in cracks in the pericarp through which microorganisms may enter. These cracks contribute to supersweet corn's predisposition to fungal diseases which adversely affect

seedling vigor. All these factors impede widespread acceptance of supersweet corn by producers, despite the market acceptance.

To satisfy both the market and the corn producers, it would be desirable to have supersweet corn which, when harvested for food at approximately 77% moisture content, typically about 19-21 days after pollination, has high sugar and low starch for flavour but has mature kernels at approximately 30-35% moisture content, about 55 days after pollination, with a starchy field corn like endosperm for ease of seed processing, improved germination and disease resistance. No such supersweet corn is currently available.

In the recent past it has been proposed to provide plants having an enhanced ability to produce starch by use of recombinant DNA techniques. Thus European patent application EP 0 368 506 (ICI) discloses a plant having enhanced ability to produce starch comprising a starch synthesizing plant having stably incorporated within its genome by transformation of one or more than one additional copy of a gene encoding ADP-glucose pyrophosphorylase. Similarly International patent application WO 91/19806 (Monsanto) concerns a method for increasing the starch content of a plant which comprises altering said plant to increase the ADP glucose pyrophosphorylase activity in said plant. Also European patent application EP 0 455 316 (IGFB) concerns plasmids which may be used to increase or decrease protein concentration or to both reduce starch concentration and increase saccharide concentration in plant cells, for instance by decreasing ADP-GPP activity. However, none of these proposals are directed towards the improvement of sweet corn seed quality.

#### DETAILED DESCRIPTION OF THE INVENTION

This invention provides corn with improved kernel characteristics such that when harvested for food it has

"supersweet" taste, but when harvested for seed, it has starchy kernels similar to those of field corn.

In a first aspect this invention provides supersweet corn which has been genetically modified to express ADP-glucose pyrophosphorylase activity at approximately 25-30 days post pollination.

In this and other aspects the invention includes genetically modified corn plants, and propagatable parts thereof including cells and tissue, and seeds, and progeny thereof including hybrid progeny thereof.

In a first embodiment, this aspect of the invention includes supersweet corn which is homozygous recessive sh-2 and contains within its genome a functional Sh-2 structural gene under the control of a heterologous promoter.

In a second embodiment this aspect also includes supersweet corn which is homozygous recessive bt-2 and contains within its genome a functional Bt-2 structural gene under the control of a heterologous promoter.

In a third embodiment this aspect also includes supersweet corn which is sh-2 and/or bt-2 homozygous recessive and contains within its genome a functional Sh-2 gene under the control of a heterologous promoter and a functional Bt-2 gene under the control of a heterologous promoter.

The heterologous promoters used in the above embodiments of the invention may comprise developmentally delayed promoters such that the Sh-2 gene and/or Bt-2 gene does not begin to be expressed until 20 to 25 days post pollination. Such corn plants are phenotypically characterized by having supersweet corn kernels at the time at harvest for food but have kernels which resemble the starchy kernels of field corn when harvested later for seed

production.

Alternatively the heterologous promoters used in this aspect of the invention may comprise inducible promoters. Thus if the plant is grown for its seed crop, it is exposed to the inducer and starch synthesis takes place. If grown for a food crop, the plant is not exposed to the inducer, so very little, if any starch synthesis takes place and the kernels are supersweet.

In a further aspect the invention also provides sweet corn which has been genetically modified by inclusion of anti-sense DNA the transcription of which is regulated or regulatable such that expression of ADP-glucose pyrophosphorylase activity is or can be inhibited until at least 25 to 30 days post pollination.

Typically in this further aspect the sweet corn contains within its genome anti-sense Sh-2 DNA under the control of a heterologous promoter or/and anti-sense Bt-2 DNA under the control of a heterologous promoter.

The heterologous promoters used may comprise inducible promoters, such that when the plant is exposed to the inducer anti-sense DNA is transcribed and blocks expression of ADP-GPP activity resulting in supersweet corn kernels. If no inducer is used with such inducible promoters, normal sweet corn results which may be harvested for food or seed as is customary for normal sweet corn. When an inducer is used the resulting supersweet corn is preferably harvested for food and is not grown on for seed harvest.

Alternatively the promoters used in this further aspect may comprise a promoter which is active in early endosperm development, i.e. a promoter which is active during the period up to 25 to 30 days post pollination. Such promoters include natural endosperm promoters which are active early in endosperm development. In addition to natural promoters, synthetic promoters

which function as early active endosperm promoters may be used. For instance, a natural or synthetic constitutive promoter element which has been manipulated to contain an early active endosperm promoter element and which is operatively linked to an element which confers endosperm activity may be used.

In a yet further aspect the invention provides a DNA expression cassette comprising a protein coding DNA sequence and a promoter wherein the DNA sequence comprises DNA coding for a corn ADP-GPP subunit (e.g. Sh-2 or Bt-2) or a functional part thereof or a DNA sequence which hybridises therewith under stringent hybridisation conditions and which codes for a protein, having the activity of an ADP-GPP subunit i.e. a protein, having ADP-GPP subunit activity, and the promoter comprises a developmentally delayed promoter or an inducible promoter.

In a still further aspect the invention also provides a DNA transcription cassette comprising an anti-sense DNA sequence complementary to a DNA sequence which codes for a peptide having ADP-GPP subunit activity, and an inducible promoter or a promoter which is active in early endosperm development.

In yet still further aspects the invention includes vectors containing the DNA expression or transcription cassettes, processes for transforming corn with the cassettes or vectors and corn plants and parts thereof (tissue and cells) transformed with the cassettes or vectors.

The invention also includes cultivation processes in which genetically modified supersweet corn or sweet corn according to the invention in which inducible promoters are used, is cultivated and exposed to a corresponding inducer as required for food or seed harvest.

Moreover the invention includes genomic clones of Sh-2 and Bt-2. In particular the invention includes the genomic clone of



Sh-2 contained in plasmid pZ01300 and the genomic clone of Bt-2 contained in plasmid pZ01301, which were deposited at the American Type Culture Collection on 2 October 1991 under accession numbers 75129 and 75130 respectively, and parts variants and analogues thereof. Most particularly the invention includes the Sh-2 genomic DNA sequence as set out in the Sequence listing as SEQ ID NO:1 and parts, variants and analogues thereof. Applicants hereby indicate that they elect the "expert solution" as regards availability of the above deposits during pendency of the EPC patent application designated herein, under the provisions of Rule 28 (4) EPC.

For the purposes of the present description and claims the following terms have the following meanings:

"Sweet corn" means Zea mays in which the genes at the sugary-1 locus are in the homozygous recessive condition.

"Supersweet corn" means Zea mays in which the shrunken-2 and/or brittle-2 genes are in the homozygous recessive condition.

"Heterologous promoter" means a promoter which does not naturally control expression of its associated structural gene, although the promoter may be of Zea mays origin.

"Sh-2", with a capital S, refers to the wild-type shrunken-2 gene.

"sh-2", with a small s, refers to a recessive shrunken-2 gene. When both copies of the shrunken-2 gene are recessive (sh-2), the result is supersweet corn.

"Bt-2", with a capital B, refers to the wild-type brittle-2 gene.

"bt-2", with a small b, refers to the recessive brittle-2 gene. When both copies of the brittle-2 gene are recessive (bt-2), the result is supersweet corn.

"Dpp" means days post pollination.

The time of "harvest for food" is generally that time when kernel moisture content is approximately 77%. Under typical environmental growing conditions, this is generally 19-21 dpp.

The time of "harvest for seed crop" is generally that time when the kernel moisture content is approximately 30-35%. Under optimal environmental growing conditions, this is approximately 55

dpp.

"Stringent hybridisation conditions" as used throughout the specification and claims are those in which hybridisation is effected in a standard manner at 65°C in 4X buffered saline (a.k.a. SSPE buffer) followed by merely washing at 57°C in 0.2X SSPE, which will not affect true duplexes which have formed.

#### DESCRIPTION OF THE FIGURES

Figure 1 shows the Northern blot for Sh-2 mRNA in two corn lines, a regular sweet corn hybrid "201 X 202" (su Sh-2) and a supersweet inbred "101" (Su sh-2). The probe used is [32P]-Sh-2 cDNA. Lanes 1-5 are "201 X 202" and lane 6 is "101". Lane 1 shows 2.5 µg endosperm bound polysomal RNA at 25 dpp; Lane 2 shows 2.5 µg free polysomal RNA at 25 dpp; Lane 3 shows 10 µg total endosperm RNA at 25 dpp; Lane 4 is 10 µg leaf total RNA; Lane 5 is 10 µg root RNA; Lane 6 is 10 µg total endosperm RNA 21 dpp.

Figure 2 is the same blot as in Figure 1 after it is stripped and subsequently probed with [32P]-labelled Bt-2 cDNA.

Figure 3 is a graph showing the developmental profile of both polysomal and total Bt-2 RNA.

Figure 4 is a graph showing the developmental profile of both polysomal and total Sh-2 RNA.

Figure 5 is a composite restriction map of the Sh-2 genomic clone.

Figure 6 is a restriction map of the Bt-2 genomic clone.

Figure 7 is a graph of ADP glucose pyrophosphorylase activity of anti-sense transformants. 17.5 µg of total soluble cellular protein was added to each assay. The assay is coupled to NADPH reduction and absorbance at 340 nm is measured.

The various aspects of the invention identified above are now described in greater detail.

#### Developmentally Delayed Promoters

It was unclear how presence of sh-2 brings about lack of ADP-GPP activity in various supersweet corn lines. Thus as a first step the biochemical manifestation of sh-2 was investigated. Polysomes from the endosperm of two corn lines (one normal sweet, one supersweet) were isolated at various days post pollination, and Northern blots were performed using either radiolabelled Sh-2 cDNA or Bt-2 cDNA. The results are shown in Figures 1 and 2. The supersweet line, designated 101 showed no Sh-2 mRNA present while the comparison line, a normal sweet corn hybrid (su Sh-2) designated (201 X 202) does express Sh-2 mRNA. When the same polysomes were re-tested using the Bt-2 probe, both lines were shown to express Bt-2 mRNA, although in (201 X 202) the amount of Bt-2 mRNA expressed is lower than the amount of Sh-2 mRNA.

One approach to modifying supersweet corn so that it retains a high sucrose concentration when harvested for food, but whose seed crop is starchy is to transfer either the Sh-2 structural gene into a homozygous sh-2 plant and/or a Bt-2 structural gene into a homozygous bt-2 plant under the control of a promoter which does not engender transcription until after harvest of the food crop. These promoters, collectively referred to as "developmentally delayed" promoters, normally control synthesis of mRNAs that appear late in the development of endosperm. Such promoters may be detected in an assay utilizing identifiable markers (such as GUS) operably linked to the putative promoter and assayed in endosperm tissue. Preferably the developmentally delayed promoter does not become active in endosperm tissue until after about 20 days post pollination (dpp). The optimum promoter for a given heterologous gene may depend on characteristics of the given gene, but should be chosen so that maximum expression and/or

gene product accumulation occurs at 25-30 dpp. Developmentally delayed promoters may be obtained using the procedures described herein.

In order to determine whether the expression of the Bt-2 and Sh-2 proteins in endosperm is due to, at least in part, a translational control mechanism, the appearance of Sh-2 and Bt-2 proteins relative to the appearance of their respective mRNAs was investigated. This information indicates when it is desirable for the developmentally delayed promoter to be active. It is assumed that the loading of mRNA onto polysomes is evidence that translation into protein is occurring. Total cellular and polysomal mRNA populations were isolated throughout endosperm development. Northern blots using radiolabelled Bt-2 and Sh-2 cDNAs probes were performed, and the results are given in Figures 3 and 4.

For Bt-2, the polysomal and total mRNA curves are virtually the same. However, the results for Sh-2, as shown in Figure 4 are somewhat different. With Sh-2, there is a peak in both total and polysomal RNA populations at 25 dpp. However, there is a lag in loading of the accumulating Sh-2 mRNA onto polysomes. This affects the choice of a developmentally delayed promoter; in order to have maximum Sh-2 expression at 25-30 dpp, the developmentally delayed promoter should preferably be most active at 20-25 dpp. For Bt-2, where no lag in polysome loading occurs, it is preferable to choose a developmentally delayed promoter which has maximum activity at 25-30 dpp for maximum expression of the Bt-2 protein at 25-30 dpp.

The actual Sh-2 and Bt-2 protein accumulations are also measured as a confirmation of the above finding. Anti-sera are made to both Sh-2 and Bt-2 proteins. Protein is isolated from the endosperm at different times post-pollination and is electrophoretically separated and blotted onto a nitrocellulose filter. The filters are probed using the appropriate anti-serum and an antibody sandwich assay is used to visualize the band. The

binding is quantified, and is found to confirm the above findings.

It is also understood that for enzymes such as ADP-GPP which are heteromultimeric, that the genetic elimination of one of the subunits (such as the Sh-2 protein) may adversely affect the stability of the other subunit(s) (such as the Bt-2 protein). Thus, depending on the nature of the particular sh-2 line, it may be necessary to provide both the Sh-2 and the Bt-2 proteins in order for the starchy phenotype to be expressed. Thus this invention also includes plants transformed with both the Sh-2 and Bt-2 genes, said genes each under the control of a heterologous promoter, and preferably each under the control of a different heterologous promoter.

In order to isolate developmentally delayed promoters, a modified subtractive technique strategy may also be used. In the modified subtractive technique (Timblin et al., 1990. Nucl. Acids Res. 18(6): 1587-1593), double stranded cDNA from a particular time point is made blunt-ended and linkers are added. The linkers serve as a PCR oligomer binding site. The cDNA is then amplified prior to cloning and can be used as a probe as well. This technique allows identification of clones which have either qualitative or quantitative differences between two different mRNA populations. Two developmentally delayed promoters are isolated, one which directs transcription beginning at approximately 20 dpp, hereinafter referred to as Promoter 20, and one which initiates transcription at approximately 25 dpp, hereinafter referred to as Promoter 25.

Genomic clones of Sh-2 and Bt-2 are made using a genomic library of maize inbred line W22 and Lambda EMBL3. (W22 is a publicly available line which can be obtained from Maize Cooperative, Stock Center, Univ. Missouri, Columbia, Missouri). The genomic clones are screened using cDNA of Sh-2 or Bt-2. Two clones are isolated for Sh-2, shown in Figure 5. A single clone is isolated for Bt-2, and this appears in Figure 6. The genomic

clones for Bt-2 and for Sh-2 comprise another aspect of this invention.

Sequencing the Sh-2 genomic clones was difficult, as standard sub-cloning techniques did not readily produce the desired subclones. Surprisingly it was found that the Sh-2 gene contains a sequence which interferes with plasmid replication. One technique which successfully produced clones, albeit at a low level (approximately 1% of the expected level) was a three-way ligation. One aliquot of vector carrying Sh-2 sequences was cut in its ampicillin resistance (amp<sup>r</sup>) gene with ScaI and in its polylinker with BamHI. A second aliquot was cut with ScaI and SalI. Fragments were isolated on gels, and a three-way ligation was performed with the Sh-2 fragment having BamHI/SalI ends, and the vector fragments such that the amp<sup>r</sup> gene is recreated upon proper re-ligation. Only bacterial clones harbouring Sh-2 DNA which have correctly re-formed were able to grow on ampicillin containing media.

The result of the sequencing of the Sh-2 gene is given in TABLE 1 (SEQ. ID. NO. 1). This sequence, containing introns, is included within the scope of this invention, as are DNA sequences which code for functional Sh-2 protein and which hybridise to the complementary strand of the sequence given in Table 1, under stringent hybridisation conditions. Expression cassettes comprising these sequences, operably linked to a heterologous promoter are also included in the invention.

For example, the following constructs may be assembled: Promoter 20-Sh-2 and Promoter 25-Bt-2. These constructs and other constructs which include other preferred developmentally delayed promoters operably linked to either the Sh-2 or the Bt-2 structural gene are specific embodiments of this invention.

#### Inducible Promoters

An alternative approach, also included within this invention, is to place the Sh-2 and/or Bt-2 genes under the control of a promoter which is not developmentally regulated, but is inducible chemically. A preferred class of inducible promoters are steroid responsive promoters. Gene expression activation by steroids such as ecdysteroid-like molecules occurs via ligand binding to protein receptors which are members of the steroid receptor superfamily. The steroid ligand binds to its cognate receptor altering receptor conformation, and the resultant binary complex recognises and binds to a steroid response element (SRE) present in the promoter to modulate transcription. For examples of such receptors, see Power et al. 1992. "New Insights into Activation of the Steroid Hormone Receptor Superfamily" *TIPS* 13:318-323; Schena, M. et al, 1991 "A Steroid-inducible Gene Expression System For Plant Cells" *Proc. Natl. Acad. Sci.* 88:10421-10425; and Fuller, P. 1991. "The Steroid Receptor Superfamily: Mechanisms of Diversity" *FASEB Journal* 5: 3092-3099, each of which is hereby incorporated by reference.

Thus, in this embodiment of the invention, the plant cell is transformed with genes encoding both the receptor and the the desired gene operably linked with a steroid responsive promoter, i.e. a natural or constructed promoter comprising an SRE. Such promoters include those which are responsive to the estrogen/estrogen receptor, progesterone/ progesterone receptor, vitamin D/ vitamin D receptor and dexamethasone/ glucocorticoid receptor complexes. Preferred promoter are those which are responsive to

the invertebrate molting hormone, 20-hydroxyecdysone (20-OHE) / receptor complex, which can be induced by the agonist compound 1,2-dibenzoyl,1-tert-butyl hydrazine. (See, e.g. Wing, et al., 1990, "Ecdysteroid Agonists as Novel Insect Growth Regulators" in Pesticides and Alternatives, Casida, Ed. Elsevier Science Publishers, p. 251-257). Thus in a preferred embodiment, the corn plant is transformed with two constructs: 1) the inducible promoter Sh-2 and/or Bt-2 construct and 2) a receptor for the inducer. Shortly before or after pollination the transformed corn is exposed to the chemical inducer. Transcription of the Sh-2 and/or Bt-2 gene is then initiated, leading to kernels with starchy endosperms. If the plant is transformed with both the Sh-2 and Bt-2 structural genes, both genes may be under the control of an inducible promoter, preferably a part of a ecdysteroid system.

In the past it had been reported that improperly folded ecdysteroid binding protein (EcRB) was produced when the gene was transfected into a number of different cell types. Thus, it is an unexpected finding in accordance with this invention that when maize cells are genetically engineered to express the EcRB gene, folding of the EcRB gene product occurs correctly, and such that the SRE is recognised and transcription is induced.

We have also observed that the ecdysteroid binding protein is capable of inducing transcription in the absence of the cognate ligand. The existence of phytoecdysteroids is documented in other species; however heretofore they were unknown in maize. It was necessary, therefore to confirm that significant levels of these materials were not present in corn since, if present they could give rise to starch production during early endosperm development which is completely contrary to supersweet corn requirements. To search for possible maize ecdysteroids, an extraction of the endosperm of maize cells was performed as described in the Examples. No significant ecdysteroid concentrations were found in BMS suspension tissue culture or maize endosperm culture.



To confirm whether such an inducible system would be operable in the corn, i.e. whether an ecdysone mimic would be taken up systemically by a growing corn plant and transported to the endosperm, the compound 322-843 (an ecdysone mimic available from Rohm and Haas as RH-5849) was radiolabelled with  $^{14}\text{C}$  for use as an internal standard for unlabelled 322-843. A residue method for detection and quantification of 322-843 was developed. A 200 mg soil drench treatment 1 or 7 days post-pollination resulted in a level of 322-843 within the endosperm which is sufficient to activate the ecdysteroid receptor. This observation was confirmed by showing that a purified extract from endosperm of treated corn triggered an ecdysteroid response in genetically engineered 20-OH ecdysone responsive Drosophila cells.

Plants which are transformed with an inducible promoter do not exhibit substantial ADP-GPP activity until presented with the inducer, after which starch synthesis and starch accumulation occurs. The progeny of these plants (either inbred or the result of a cross with a non-related, transformed or non-transformed plant) inducibly express the starchy phenotype. All of these progeny plants are included within the invention.

#### Antisense Constructs

In yet another aspect of this invention, an anti-sense transcription construct can be used to regulate starch synthesis. The Sh-2 gene or the Sh-2 cDNA in its anti-sense configuration is placed under the control of an inducible promoter, such as those described above or an endosperm promoter active early in endosperm development. Alternatively, a synthetic promoter can be made which contains an early active endosperm promoter element operatively linked to an element which confers endosperm activity. Examples of promoters which contain early active endosperm elements include 1) various zein promoters, such as the promoter for gamma, beta or alpha zein; 2) a 198 base pair portion of the

low molecular weight glutenin (LMWG) promoter (as in Colot et al, 1987 EMBO J. 6(12):355-359, which is hereby incorporated by reference); and 3) a 227 base pair portion of the high molecular weight wheat glutenin (HMWG) promoter (as in Thomas and Flavell The Plant Cell 2:1171, which is hereby incorporated by reference).

The aforementioned elements may be operatively linked to a normally constitutive promoter element such as the minimal 35S promoter (i.e. comprising the -46 base pair upstream region) or the minimal HSP82 promoter, such as described in co-pending application serial number 07/791,921 which is incorporated by reference.

A normal sweet corn plant (su Sh-2) is transformed with one of the aforementioned constructs. When exposed to the inducer, anti-sense Sh-2 mRNA is transcribed which hybridizes with the wild type Sh-2 mRNA, thereby blocking synthesis of the Sh-2 protein. Consequently, starch synthesis is impaired and sucrose accumulates. As can be seen by one of ordinary skill in the art, this anti-sense approach can also be used with the Bt-2 gene.

Typically the promoter constructs used for the expression and transcription constructs, whether they are developmentally regulated or inducible, also contain other DNA sequences, such as enhancers and 3' termination sequences. Such sequences are well known in the art. The cloned construct is then inserted into a plasmid or other vector suitable for transformation.

Maize type II friable callus, suitable for transformation and regeneration is cultured using known techniques (Green, L.E. et al. 1975. "Plant Regeneration From Tissue Cultures of Maize." Crop Science 15:417-421 and Vasil, V. et al. 1984. "Somatic Embryogenesis in Long-term Callus Cultures of Zea mays L. (Gramineae)" Amer. J. Bot. 71:158-61, both of which are incorporated herein by reference). Cells are transformed using the ballistic technique, detailed below in the Examples. Putative

transformants are regenerated into intact plants. The plants which have been transformed with a developmentally delayed promoter construct are grown to maturity and are seen to bear supersweet corn which later (approximately 25-30 dpp) becomes starchy. The progeny of these plants (either inbred or the result of a cross with a non-related, transformed or non-transformed plant) also bear supersweet corn which ages into starchy corn. All of these progeny plants are included within the invention.

The following deposits were made at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 on October 22, 1991 in accordance with the provisions of the Budapest Treaty: pZ01301, containing the Bt-2 genomic clone which received ATCC number 75130; and pZ01300, containing the Sh-2 genomic clone which received ATCC number 75129.

The following, non-limiting Examples are presented to better illustrate the invention.

## DESCRIPTION OF EMBODIMENTS OF THE INVENTION

## EXAMPLE 1

## POLYSOMAL RNA ISOLATION

Polysomes are isolated according to the methods of Larkins et al. 1976 "Isolation and in Vitro Translation of Zein Messenger Ribonucleic Acid" Biochem 75 (25): 5586, Larkins et al. 1976. "Storage Protein Synthesis in Maize" Plant Physiol 57:740-745, and Larkins et al. 1978. "Synthesis and Deposition of Zein in Protein Bodies of Maize Endosperm" Plant Physiol. 62:256-263, which are hereby incorporated by reference. Isolated polysomes are resuspended in 1% w/v triisopropyl-naphthalene-sulfonic acid, Na salt; 6% w/v p-amino-salicylic acid; 0.1 M TRIS-HCl, pH 7.6; 50 mM EGTA; 0.1 M NaCl; 1% w/v SDS; and 50 mM 2-mercaptoethanol (Rochester et al. 1986. EMBO J. 5:451-458). An equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) is added. After centrifugation, the supernatant fluid is removed to a new tube and re-extracted. The aqueous phase is centrifuged at 50,000 rpm in a Beckman SW55Ti rotor for 30 minutes at 4°C. The supernatant is then ethanol precipitated by the addition of 0.1 volume 3M NaOAc and 2.5 volumes of ethanol. The pellet is resuspended in 750 µl for every 20 g endosperm originally ground. To this, 300 µl of 10M LiCl are added and H<sub>2</sub>O to a final volume of 1.5 ml. After 1 hour at 4°C, RNA is pelleted at 14,000 X g for 30 minutes. The pellet is resuspended in 400 µl H<sub>2</sub>O and centrifuged in a microfuge to remove any insoluble material. The supernatant is removed and 100 µl LiCl is added. This is incubated and RNA is collected as above.

## EXAMPLE 2

ISOLATION AND CHARACTERIZATION OF GENOMIC  
CLONES FOR Bt-2 AND Sh-2

cDNA clones for Sh-2 and Bt-2 are isolated as reported by

Bhave et al., 1990, "Identification and Molecular Characterization of Shrunk-2 cDNA Clones of Maize" The Plant Cell 2:581-588, and Bae et al., 1990, Maydica 35:317-322, both of which are incorporated by reference. A genomic library from public maize line W22 is made in Lambda EMBL3 according to standard procedures as described in Ausubel et al. 1987. Current Protocols in Biology, Wiley Interscience. Approximately  $5 \times 10^6$  clones are transferred to nitrocellulose and are screened using the radiolabelled cDNAs as probes, in 5X SSC, 0.1% SDS, 1X Denhardt's solution, 50% formamide and 42°C for hybridization. Filters are washed four times for 30 minutes at 65°C in 0.1X SSC and 0.1% SDS.

Genomic clones isolated in this way are mapped first using Lambda Map (Promega). The rough map generated is then refined using conventional mapping techniques of probing Southern blots of various restriction digests of the clones. Maps thus generated are shown in Figures 5-6. Subcloning of the genomic clones is done with pT7T3 vectors (Pharmacia). Nested deletions are performed with a commercial kit according to the directions of the manufacturer (Pharmacia). Sequencing is done by the dideoxy chain termination method using T7 polymerase (Pharmacia). Sequences of the Sh-2 genomic clone is given in TABLE 1 (SEQ.ID NO:1).

### EXAMPLE 3

#### GENERATION OF ANTI-SERA TO Bt-2 AND Sh-2 POLYPEPTIDES

Portions of the cDNAs corresponding to Bt-2 and Sh-2 are cloned into the E. coli expression vector pGEX 2T (Pharmacia). This produces glutathione-S-transferase (GST) fusions that have a thrombin protease recognition site interposed between the GST moiety and the maize protein. Fusion proteins are produced by induction of the pGEX 2T ptac promoter by the addition of IPTG. Fusion proteins are affinity purified on a glutathione-sepharose column according to the directions of the manufacturer. Purified fusion proteins are cleaved by thrombin, repassed over the glutathione-sepharose column and the void volume is collected.

Rabbits are immunized with 650-850 µg of either of the purified proteins in complete Freund's adjuvant and boosted every other week with 250 µg of protein in incomplete Freund's adjuvant. Anti-Bt-2 and Anti-Sh-2 sera is obtained.

#### EXAMPLE 4

##### PROMOTER IDENTIFICATION, ISOLATION AND TESTING OF DEVELOPMENTALLY DELAYED PROMOTERS

Modified subtractive cDNA libraries are made using the procedure of Timblin et al.(1990 Nucl. Acids Res. 18(6):1587-1593) which is hereby incorporated by reference. mRNA populations are isolated 15, 25, and 35 dpp. Double stranded cDNAs from these populations are made blunt and linkers are added. The linkers are chosen so as to serve as a Polymerase Chain Reaction (PCR) oligomer binding site. The resulting cDNAs are amplified according to the manufacturer's recommended instructions and are also used as probes. Clones are identified which show differences (either quantitative or qualitative) in the mRNA populations. These represent potential developmentally delayed promoters. The copy numbers of these clones are determined using standard protocols, and those with a low copy number (<5 copies per genome) are selected. These are used for in situ hybridization to maize endosperm sections to determine which are active in appropriate cell layers.

Using the selected cDNA clones which have both low copy number and correct spatial expression, genes are isolated from a maize genomic library. Promoter regions are isolated using conventional techniques. Putative promoters are tested by constructing b-glucuronidase (GUS) fusions, delivering the hybrid construct to longitudinal endosperm sections via the ballistic particle delivery process and visually determining their activity by GUS staining (Jefferson, R.A. 1988. in Genetic Engineering, Principles and Methods, Vol. 10:247-263 J.K. Setlow, Ed.)

The promoters resulting from this isolation and evaluation are designated Promoter 20, which maximizes mRNA synthesis at approximately 20 dpp and Promoter 30 which maximizes mRNA synthesis at approximately 30 dpp. These are used to make transformation vectors, discussed in Example 7, below.

EXAMPLE 5  
IDENTIFICATION OF DEVELOPMENTALLY DELAYED PROMOTERS  
USING POLYSOMAL RNA

The procedure of Example 4 is repeated, using polysomal RNA. The same developmentally delayed promoters are identified.

EXAMPLE 6  
CONSTRUCTION OF VECTORS FOR CALLUS TRANSFORMATION

The following transformation vectors are constructed. Promoter 20, from Example 5 is linked to the Sh-2 gene, along with the Sh-2 gene endogenous terminator. This construct is referred to as the agronomic cassette-20. A similar cassette is constructed using Promoter 30 linked to the Bt-2 gene and terminator, referred to as agronomic cassette-30.

The vectors used for maize callus transformation are pUC19 derivatives a bacterial selectable marker ( $\beta$ -lactamase, i.e. ampicillin/carbenicillin resistance) and a maize selectable marker. The maize selectable marker has the cauliflower mosaic virus 35S promoter, a portion of intervening sequence II from maize alcohol dehydrogenase, and a selectable gene: neomycin phosphotransferase II (i.e. kanamycin resistance) or phosphinothricin acetyltransferase (i.e. resistance to the herbicides Basta and Bialophos) or acetolactate synthase (i.e. resistance to the herbicide chlorsulfuron). The selectable gene sequence is followed by the nopalene synthase terminator.

The agronomic cassette-20 is inserted using standard

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techniques into the above vector to create pSh-2. Similarly agronomic cassette-30 is inserted into the vector to create pBt-2. E. coli are transformed with either plasmid using standard techniques (Ausabel et al., supra).

## EXAMPLE 7

INITIATION AND MAINTENANCE OF TYPE II  
FRIABLE MAIZE CALLUS

1.5 mm embryos (10-13 dpp) from 29 sh-2 lines are explanted and placed on initiation medium (either Murashige-Skoog with 1.5 mg/l Dicamba or N6 with 1 mg/l 2,4-D). Callus is transferred to and maintained on N6 containing 6 mM asparagine, 12 mM proline and 1 mg/l 2,4-D. Friable callus is selected and is used in the transformation procedure, below.

## EXAMPLE 8.

GENERATION OF TRANSGENIC PLANTS AND  
TESTING THE DELAYED EXPRESSION OF Sh-2 or Bt-2

Plasmid pSh-2 is introduced into Type II maize callus of a sh-2 supersweet corn line using a ballistic process. Plants are regenerated following known procedures and are self-pollinated. ADP-GPP activity is determined prior to 20 dpp and after 30 dpp to ascertain the induction of Sh-2 transcription and its subsequent translation. Plants are then evaluated for agronomic performance, and those with the desired phenotype are identified.

Plasmid pBt-2 is introduced into Type II maize callus of a bt-2 line using a ballistic process. Plants are regenerated following known procedures and are self-pollinated. ADP-GPP activity is determined prior to 20 dpp and after 30 dpp to ascertain the induction of Bt-2 transcription and translation. Plants are then evaluated for agronomic performance, and those with the desired phenotype are selected.



EXAMPLE 9  
INDUCIBLE PROMOTER CONSTRUCTS

Vectors for callus transformation are constructed similarly to those described in Example 6, except for the agronomic cassettes. Here, the agronomic cassette contains the promoter which is inducible by the 20-hydroxyecdysone binding protein binary complex operably linked to either the Sh-2 gene or Bt-2 gene and their respective endogenous terminators. The plasmid containing the inducible Sh-2 construct is designated pISh-2. The plasmid containing the inducible Bt-2 construct is designated pIBt-2.

Maize calli are co-transformed with either pISh-2 and pRecept or pIBt-2 and pRecept. pRecept is a compatible plasmid which codes for expression of the 20-hydroxyecdysone receptor. Transformants carrying both heterologous gene constructs are selected and regenerated into plants which inducibly express either Sh-2 or Bt-2.

Additional constructs are as follows (5' to 3').

Construct 1110 contains the 35S promoter, the maize IVS6 intron, EcRB gene construct and the Nos terminator.

Construct 1112 comprises the Steroid Response Element (SRE) linked to the -46 35S promoter fragment (base pairs -46 to +1), the maize IVS6 intron, GUS gene and the NOS terminator.

Construct 1113 comprises the SRE (as above) linked to a maize promoter, the IVS6 intron, the gene and the NOS terminator.

Black Mexican Sweet (BMS) suspension cells are transformed with the above constructs using the ballistic technique, incubated overnight, and then are overlaid with X-gluc staining solution

which may contain 1  $\mu$ M of the phytoecdysteroid Ponasterone A (PNA), as indicated below. Three plates are transformed for each construct. Results are presented below.

Construct	PNA	# Blue Stains
1112	+	2, 4, 2
1110 + 1112	+	1240, 600, 1300

In the following experiment, cells are transformed as above, then scraped off the filter paper and incubated overnight in an MS solution in the presence or absence of 1  $\mu$ M PNA. The next morning, cells are x-gluc stained. Results are presented below.

Construct	PNA	# Blue Stains
1110	-	0, 0, 0
1110	+	0, 0, 0
1112	-	45, 16, 27
1112	+	35, 32, 45
1110 + 1112	-	490, 833, 755
1110 + 1112	+	456, 490, 441

In this experiment, cells are transformed and then are overlaid with MS medium in the presence or absence of 1  $\mu$ M PNA overnight. The next morning, they are stained with X-gluc. Results are presented below.

Construct	PNA	# Blue Stains
1110	-	8, 8, 5
1110	+	4, 4, 9
1113	-	6, 2, 3
1113	+	5, 3, 2
1110 + 1113	-	261, 26, 75
1110 + 1113	+	256, 210, 468

#### EXAMPLE 10

##### Isolation of Ecdysteroids From Maize

A residue method is used which is a modification of that of Horn and Bergamasco (1985, Comprehensive Insect Physi l. Biochem.

Pharmacol. Kerkut and Gilbert, Eds. (7):212) which is hereby incorporated by reference. 3-5 g aliquots of Black Mexican Sweet (BMS) maize cells from suspension or frozen endosperm tissue from a sweet corn line, were processed in a Polytron homogeniser with 200 ml 96% ethanol. The extract is dried in a vacuum and the residue is partitioned between hexane and 75% ethanol (10 ml each phase). The ethanol phase is then concentrated in a vacuum and partitioned between methylene chloride: ethanol: water (2 ml each phase), and the methylene chloride phase is then concentrated to dryness. This is dissolved in ethyl acetate: ethanol (2:1) as a 5% solution and filtered through neutral alumina (10% H<sub>2</sub>O, 2 g) and is eluted with further solvent (25 ml). The total eluate is evaporated to dryness. The crude ecdysteroids are then dissolved in methylene chloride: ethanol (2:1 to make a 5% solution w/v) and an aliquot is transferred to a TLC plate for purification.

The fractions are tested for ecdysone agonist activity by incubation with a genetically engineered *Drosophila* cell line which is ecdysone responsive. No ecdysone activity is observed in either the sweet corn endosperm or BMS cell extracts, with a limit of detection <20 ng 20-OH-ecdysone equivalents/g. Hexane (lipophilic) and aqueous/ethanol (polar) fractions are also investigated for ecdysone agonist activity since transfection assays with the insect ecdysone receptor/hormone response element genes incorporated into BMS cells have indicated an endogenous supply of ecdysone agonist positive substance. The lipophilic and polar fractions are assayed in the ecdysone responsive cell line. No activity is observed, but some toxicity is noted in the concentrated polar samples. However, it can be calculated that the level of 20-OH-ecdysone equivalents is <200 ng/g endosperm or <100 ng/g BMS cells, and may be much less.

#### EXAMPLE 11

##### SOIL DRENCH AND FOLIAR SPRAY

###### A. Soil Drench Test

Three dosages of 322-843 are prepared: 0, 20 mg and 200 mg

active ingredient per plant. For the 0 mg dose, 100 ml dimethylsulfoxide (DMSO) is added to 20 liters of water in a clean container and is mixed thoroughly. Four liters are applied to each plant a 1, 7, or 14 days post pollination. Each plant is in a 5 gallon container fitted with a base to catch leachate so that the leachate can be reabsorbed into the soil. For the 20 mg dose, the same procedures are followed except that 0.1 g 322-843 is dissolved in the 100 ml DMSO. For the 200 mg dose, 1.0 g of 322-843 is dissolved in the DMSO. Regardless of the dose, all solutions are prepared just prior to use. Ears are harvested 16 days post-pollination and are frozen in liquid nitrogen.

#### B. Foliar Spray

Three dosages of 322-843 (0, 20, and 200 mg active ingredient per plant) are prepared as below just prior to application at 1, 7, or 14 days post pollination. Ears are harvested at 16 days post-pollination and are frozen in liquid nitrogen. For 0 mg, 2.5 g Valent X-77 is dissolved in 500 ml acetone to make a 0.5% solution. 100 ml of this solution is mixed with 100 ml water. 40 ml of the solution are sprayed per plant. For 20 mg, 0.1 g 322-843 is dissolved in 100 ml of the 0.5% Valent X-77/acetone solution and then mixed with 100ml water. 40 ml are sprayed per plant. For 200 mg, 1.0 g 322-843 is dissolved in 100 ml of the 0.5% Valent X-77/acetone solution and mixed with 100 ml water. 40 ml are sprayed per plant.

Endosperm from plants treated by the soil drench or foliar spray is analyzed as follows. For a standard, 10  $\mu$ l of a  $\text{CH}_3\text{CN}$  solution of  $[\text{14C}]$ 322-843 containing approximately 25,000 dpm (approximately 30 ng) is dispensed into 25 ml methanol in a 50 ml beaker. 3 g of heat-treated celite and 5 g of thawed endosperm are added, and then chilled over ice. (The blank does not contain any corn tissue). The corn tissue is homogenized, and the homogenate is filtered under a vacuum. The filter cake is returned to the original flask and 25 ml dichloromethane is added, and the procedure is repeated.

The solvent is then removed under a vacuum and mild heat, leaving an oily residue. The residue is dissolved in 1-2 ml dichloromethane and water. The solvent layer is removed, and the contents are extracted with 2 x 100 µl of acetonitrile and then dried. The acetonitrile extract is re-dissolved in 50 µl dichloromethane and vortexed. A sample is added to a silica GF TLC plate (1000 µl thick layer) which is predeveloped with methanol and air dried for 24 hours. The solvent is evaporated from the plate and plates are developed in 3% methanol/97% dichloromethane. Plates are air-dried and scanned with a radioactivity monitor. The radioactive zone is transferred to a fritted funnel and the 322-843 is eluted with acetonitrile under a slight vacuum. The sample is redissolved in acetonitrile for quantification of recovery and subsequent HPLC analysis. Alternatively, it is redissolved in 50 µl DMSO for screening in the *Drosophila* ecdysone agonist insect cell line assay or HPLC analysis.

HPLC analysis is performed using a 25 x 0.46 cm Spheri-5 RP-8 column and a linear gradient system: initially 40% acetonitrile in 0.01 TFA and, after 20 minutes 90% acetonitrile in 0.01% TFA, with a 5 min hold at 90% acetonitrile and a 5 min return to starting conditions. The flow rate of solvent is 1.5 ml/min. RT for 322-843 is approximately 7.6 min. The 322-843 zone is collected for liquid scintillation counting and the UV peak area (255 nm) is integrated. After correcting for 100% recovery of the radiolabelled internal standard, total mass of 322-843 is calculated. Additional HPLC using a normal phase system was also utilised in further analysis of the samples.

Results are presented below. OP is open pollination; SP is self pollination.

Treatment	µg 322-843 per gm corn	Mass of corn endosperm (g)
1 day, soil, control	0	1.5

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1 day, soil, 200 mg	1.05	2.7
1 day, soil, 200 mg	0.90*	2.4
1 day, soil, 20 mg	0.19	4.1
1 day, soil, 20 mg	0.19	4.3
1 day, foliar, control	0.04	3.7
1 day, foliar, 200 mg (OP)	0.02	3.2
1 day, foliar, 200 mg	0.03	3.1
7 day, soil, control	0	5.1
7 day, soil, control	0.02	1.4
7 day, soil, 200 mg (OP)	0.56	3.7
7 day, foliar, 200 mg SP	0.14	3.4
14 day, soil, 200 mg SP	0.25	5.1
14 day foliar, control	0.28	0.50
14 day, foliar, 200 mg	0.06	0.15

\*Purified product gives positive response in *Drosophila* cell-line assay.

Thus, a soil application at 200 mg is sufficient for uptake of 322-843 and transport to endosperm in quantities sufficient for inducible activity.

## EXAMPLE 12

## Endosperm Specific Gene Expression

Various constructs are made using conventional cloning techniques and are tested for endosperm activity using a GUS reporter gene. The number of blue stains were counted and the results obtained are given below for the constructs tested.

Construct	Endosperm	Leaf
Full 35S	24	62
minimal 35S	1	0
LMWG/35S	5.7	0

HMWG/35S	2.3	0
minimal HSP	0	0
LMWG/HSP	7.7	0
HMWG/HSP	3.3	1.3

EXAMPLE 13  
ANTISENSE

Three different clones of an endosperm culture, 1818-2, 1618-3, and 1618-4 transformed with the Sh-2 antisense cDNA (plasmid p1618) are prepared and verified by NPTII ELISA assays. ADP glucose pyrophosphorylase assays are performed on each transformed line and compared to results of the untransformed culture from the same line (636). The results are given in Figure 7, and as can be seen the Sh-2 anti-sense cDNA significantly diminishes ADP glucose pyrophosphorylase activity.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Nichols, Scott E.  
Pauly, Michael  
Weeks, Donald  
Sinibaldi, Ralph M.  
Fred C. Baker  
Marian L. Duncan

(ii) TITLE OF INVENTION: Improved Supersweet Corn

(iii) NUMBER OF SEQUENCES: 1

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9824 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTTTAA AAGTTTCTAA TGTGAATTGT TTTTCTTTTA ACTTAAATAC TCATTCAGCG	60
AAAGGTCCTC TAGCGTAATG GGTAAGGATT CCGAGTATCA CCTCTAGGTT CTGGGTTCGA	120
CCTCATCGGG GCGGAATTTC GGGCTTGGTT AAAAAAATA CTCTCACTGT GCCCCGCCC	180
CTCCCGGGTT ACGTTCTGCG CGCCACCCTC TGGCTGGGCT GTTGTAAGT GGGCGATGAC	240
GGCCCCGCTAG TGATGGGGGG CAGGGGTGGA CCAAGTACG GCTGAGTGAG GGCACATGCC	300
CCCGCCCATT TCTTTGTTCT AAGTACTAGG GTCTAAATTT TCACCATGAG GCCCCCTGCT	360
TAGTCTAATT TAGATGCCCT TGCTCTGGTA TTTTGATGCT CGTCCTTACA AGTATGCCCT	420
TAGTCATATT TTGTCCTGGG TCCGCCCTG ATGAGAGGGG CCAGGGTTTA GAGATTTTCT	480
CGGCCGGGAC CAATGTTCTG GTCTCTTCTT AATATAATAC CGGGACAGTC TTTCCCTCTC	540
CGGCCGAGTT TTTTATACTC ACTCTAGGTG CAAACGTAAG TTGTTCAGGC TTGTGCTTAA	600



GGATTAAGAA AGTAAATCAA ATGACCATGT TACACTTTTT TTATTTATAC CGTATCGGAA	660
AGATAAACCA TTTATCTGTG AGTGGTAGCA TGGTCAAGGA GAAAAAATA GATGCATAGA	720
GGTTCCTGGGA CGACTCACAT TTAGAGCATA GTTGAAAAGG CTAAAACAAC CAGGAGGGGG	780
TGGGGTCGGG ATACATAAAT TTTCATGAGT GATGGTTGAA CTATTGAGGG TGCAAAATAA	840
TTTTTTCAAC AAATAATGTG GTGAAATACC TAAGAGGGGT GCACCTAGCA TAGATTTTTT	900
GGGGCTCCCC TTGGCCTCTC CTTTCTTCCG CCCTGAAAAC AACCTACATG GATACATCTG	960
CAACCAGAGG GAGTATCTGA TGCTTTTTTC TGGGCAGGGA GAGCTATGAG GCGTATGTCC	1020
TCAAAGCCAC TTTGCATTGT GTGAAACCAA TATCGATCTT TGTTACTTCA TCATGCGTGA	1080
ACATTTGTGG AAACCTACTAG CTTACAAGCA TTAGTGACAG CTCAGAAAAA AGTTATCTCT	1140
GAAAGGTTTC ATGTGTACCG TGGGAAATGA GAAATGTTGC CAACTCAAAC ACCTTCAATA	1200
TGTTGTTTGC AGGCAAACTC TTCTGGAAGA AAGGTGTCTA AAACCTATGAA CGGGTTACAG	1260
AAAGGTATAA ACCACGGCTG TGCATTTTGG AAGTATCATC TATAGATGTC TGTTGAGGGG	1320
AAAGCCGTAC GCCAACGTTA TTTACTCAGA AACAGCTTCA ACACACAGTT GTCTGCTTTA	1380
TGATGGCATC TCCACCCAGG CACCCACCAT CACCTATCTC TCGTGCCTGT TTATTTTCTT	1440
GCCCTTTCTG ATCATAAAAA ATCATTAAAGA GTTTGCAAAC ATGCATAGGC ATATCAATAA	1500
TTCAATATGC TCATTIATTA ATTTGCTAGC AGATCATCTT CCTACTCTTT ACTTTATTTA	1560
TTGTTTGAAA AATATGTCCT GCACCTAGGG AGCTCGTATA CAGTACCAAT GCATCTTCAT	1620
TAAATGTGAA TTTCAGAAAG GAAGTAGGAA CCTATGAGAG TATTTTTCAA AATTAATTAG	1680
CGGCTTCTAT TATGTTTATA GCAAAGGCCA AGGGCAAAAT TGGAACACTA ATGATGGTTG	1740
GTTGCATGAG TCTGTGATT ACTTGCAAGA AATGTGAACC TTTGTTTCTG TGCGTGGGCA	1800
TAAAACAAAC AGCTTCTAGC CTCTTTTACG GTACTTGAC T TGCAAGAAA TGTGAACTCC	1860
TTTTCATTTT TGTATGTGGA CATAATGCCA AAGCATCCAG GCTTTTTTCAT GGTTGTTGAT	1920
GTCTTTACAC AGTTCATCTC CACCAGTATG CCCTCCTCAT ACTCTATATA AACACATCAA	1980
CAGCATCGCA ATTAGCCACA AGATCACTTC GGGAGGCAAG TGCGATTTTG ATCTTGCAGC	2040
CACCTTTTTT TGTTCTGTTG TAAGTATACT TTCCCTTACC ATCTTTATCT GTTAGTTTAA	2100
TTTGTAATTG GGAAGTATTA GTGAAAGAG GATGAGATGC TATCATCTAT GTACTCTGCA	2160
AATGCATCTG ACGTTATATG AGCTGCTTCA TATAATTTGA ATTGCTCCAT TCTTGCCGAC	2220

AATATATTGC AAGGTATATG CCTAGTTCCA TCAAAAGTTC TGTTTTTTCA TTCTAAAAGC	2280
ATTTTAGTGG CACACAATTT TTGTCCATGA GGGAAAGGGA ATCTGTTTTG GTTACTTTGC	2340
TTGAGGTGCA TTCTTCATAT GTCCAGTTTT ATGGAAGTAA TAAACTTCAG TTTGGTCATA	2400
AGATGTCATA TTAAAGGGCA AACATATATT CAATGTTCAA TTCATCGTAA ATGTTCCCTT	2460
TTTGTA AAAAG ATTGCATACT CATTATTTTG AGTTGCAGGT GTATCTAGTA GTTGGAGGAG	2520
ATATGCAGTT TGCACTTGCA TTGGACACGA ACTCAGGTCC TCACCAGATA AGATCTTG TG	2580
AGGGTGATGG GATTGACAGG TTGGAAAAAT TAAGTATTGG GGGCAGAAAAG CAGGAGAAAAG	2640
CTTTGAGAAA TAGGTGCTTT GGTGGTAGAG TTGCTGCAAC TACACAATGT ATTCTTACCT	2700
CAGATGCTTG TCCTGAAACT CTTGTAAGTA TCCACCTCAA TTATTACTCT TACATGTTGG	2760
TTTACTTTAC GTTTGTCTTT TCAAGGGAAA TTTACTGTAT TTTTGTGTT TTGTGGGAGT	2820
TCTATACTTC TGTTGGACTG GTTATTGTAA AGATTTGTTC AAATAGGGTC ATCTTATAAT	2880
TGTTTGAAAT CTGGGAACTG TGGTTTCACT GCGTTCAGGA AAAAGTGAAT TCTTGTTAC	2940
TGCATGAATA ACTTATGGAA ATAGACCTTA GAGTTGCTGC ATGATTATCA CAAATCATTG	3000
CTACGATATC TTATAATAGT TCTTTCGACC TCGCATTACA TATATAACTG CAACTCGTAG	3060
TTGCGTTAAA AAAAATGCAA CTCTTAGAAC GTTCACCAGT GTAATCTTTC CTGAATTGTT	3120
ATTTAATGGC ATGTATGCAC TACTTGTATA CTTATCTAGG ATTAAGTAAT CTAACCTCTAG	3180
GCCCCATATT TGCAGCATT CCAAACACAG TCCTCTAGGA AAAATTATGC TGATGCAAAC	3240
CGTGTATCTG CTATCATTTT GGGCGGAGGC ACTGGATCTC AGCTCTTTCC TCTGACAAGC	3300
ACAAGAGCTA CGCCTGCTGT AAGGGATAAC ACTGAACATC CAACGTTGAT TACTCTATTA	3360
TAGTATTATA CAGACTGTAC TTTTCGAATT TATCTTAGTT TTCTACAATA TTTAGTGGAT	3420
TCTTCTCATT TTCAAGATAC ACAATTGAAC CATAATCGAA GTGGTATGTA AGACAGTGAG	3480
TTAAAAGATT ATATTTTTTG GGAGACTTCC AGTCAAATTT TCTTAGAAGT TTTTTTGTC	3540
CAGATGTTCA TAAAGTCGCC GCTTTCATAC TTTTTTTAAT TTTTAAATTG GTGCACTATT	3600
AGGTACCTGT TGGAGGATGT TACAGGCTTA TTGATATCCC TATGAGTAAC TGCTTCAACA	3660
GTGGTATAAA TAAGATATTT GTGATGAGTC AGTTCAATTC TACTTCGCTT AACCGCCATA	3720
TTCATCGTAC ATACCTTGAA GGCGGGATCA ACTTTGCTGA TGGATCTGTA CAGGTGATTT	3780
ACCTCATCTT GTTGATATGT AATACTGTAA TTAGGAGTAG ATTTGTGTGG AGAGAATAAT	3840

AAACAGATGC	CGAGATTCTT	CTCTAAAAGT	CTAGATCCAA	AGGCATTGTG	GTTCAAAACA	3900
CTATGGACTT	CTACCATTTA	TGTTATTACT	TTGCCTTAAT	GTTCCATTGA	ATAGGGCAAA	3960
TTATTGATTC	TACAAGTGTT	TAATTAATAA	CTAATTGTTC	ATCCTGCAGG	TATTAGCGGC	4020
TACACAAATG	CCTGAAGAGC	CAGCTGGATG	GTTCCAGGGT	ACAGCAGACT	CTATCAGAAA	4080
ATTTATCTGG	GTAATCGAGG	TAGTTGATAT	TTTCTCGTTT	ATGAATGTCC	ATTCATCAT	4140
TCCTGTAGCA	TTGTTTCTTT	GTAATTTTGA	GTTCTCCTGT	ATTTCTTTAG	GATTATTACA	4200
GTCACAAATC	CATTGACAAC	ATTGTAATCT	TGAGTGGCGA	TCAGCTTTAT	CGGATGAATT	4260
ACATGGAAC	TGTGCAGGTA	TGGTGTCTC	TTGTTCTCTA	TGTTTCACGT	AATGTCCTGA	4320
TTTTGGATTA	ACCAACTACT	TTTGGCATGC	ATTATTTCCA	GAAACATGTC	GAGGACGATG	4380
CTGATATCAC	TATATCATGT	GCTCCTGTTG	ATGAGAGGTA	ATCAGTTGTT	TATATCATCC	4440
TAATATGAAT	ATGTCATCTT	GTTATCCAAC	ACAGGATGCA	TATGGTCTAA	TCTGCTTTCC	4500
TTTTTTCCCT	TCGGAAGCCG	AGCTTCTAAA	AATGGGCTAG	TGAAGATTGA	TCATACTGGA	4560
CGTGTACTTC	AATTCTTTGA	AAAACCAAAG	GGTGCTGATT	TGAATTCTAT	GGTTAGAAAT	4620
TCCTTGIGTA	ATCCAATTCT	TTTGTTTTCC	TTTCTTTCTT	GAGATGAACC	CCTCTTTTAG	4680
TTATTTCCAT	GGATAACCTG	TACTTGACTT	ATTCAGAAAT	GATTTTCTAT	TTTGCTGTAG	4740
AATCTGACAC	TAAAGCTAAT	AGCTACTGAT	GTTGCAGAGA	GTTGAGACCA	ACTTCCTGAG	4800
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## CLAIMS:

1. Corn which has supersweet kernels at the time of harvest for food, and which has starchy kernels at the time of harvest for seed.

2. Supersweet corn according to claim 1, which has been genetically modified to express ADP-glucose phosphorylase (ADP-GPP) activity at approximately 25-30 days post pollination.

3. Supersweet corn according to claim 2 which is homozygous recessive *sh-2*, and contains within its genome a functional *Sh-2* gene under the control of a heterologous promoter.

4. Supersweet corn according to claim 2 which is homozygous recessive *bt-2*, and contains within its genome a functional *Bt-2* gene under the control of a heterologous promoter.

5. Supersweet corn according to claim 2 which is *sh-2* and/or *bt-2* homozygous recessive and contains within its genome a functional *Sh-2* gene under the control of a heterologous promoter and a functional *Bt-2* gene under the control of a heterologous promoter.

6. Supersweet corn according to any one of claims 3-5 wherein the heterologous promoter is a developmentally delayed promoter.

7. Supersweet corn plant according to any one of claims 3-5 wherein the heterologous promoter is an inducible promoter.

8. Sweet corn according to claim 1 which has been genetically modified by inclusion of anti-sense DNA the transcription of which is regulated or regulatable such that expression of ADP-glucose pyrophosphorylase activity is or can be inhibited until at least 25 to 30 days post pollination.

9. Sweet corn according to claim 8 which contains within its genome anti-sense Sh-2 DNA under the control of a heterologous promoter or/and anti-sense Bt-2 DNA under the control of a heterologous promoter.

10. Sweet corn according to claim 9 wherein the heterologous promoter is an inducible promoter.

11. Sweet corn according to claim 9 wherein the heterologous promoter is active in early endosperm development.

12. A supersweet or sweet corn plant according to any of the preceding claims.

13. Corn seed according to any of Claims 1 to 11.



14. A DNA expression cassette comprising a protein coding DNA sequence and a promoter, wherein the DNA sequence comprises DNA coding for a protein having the activity of a corn ADP-GPP subunit and the promoter comprises a developmentally delayed promoter or an inducible promoter.

15. A DNA transcription cassette comprising an anti-sense DNA sequence complementary to a DNA sequence which codes for a peptide having the activity of a corn ADP-GPP submit and an inducible promoter or a promoter which is active during early endosperm development.

16. A vector containing a DNA cassette according to claim 14 or 15.

17. A process for the transformation of corn in which the corn is transformed with a DNA cassette according to claim 14 or 15 or a vector according to claim 16.

18. Corn when transformed by a process according to claim 17.

19. Progeny of corn according to claim 18.

20. A cultivation process in which supersweet corn according to claim 7 or sweet corn according to claim 10 is cultivated and exposed to an inducer at the time required for food or seed

harvest.

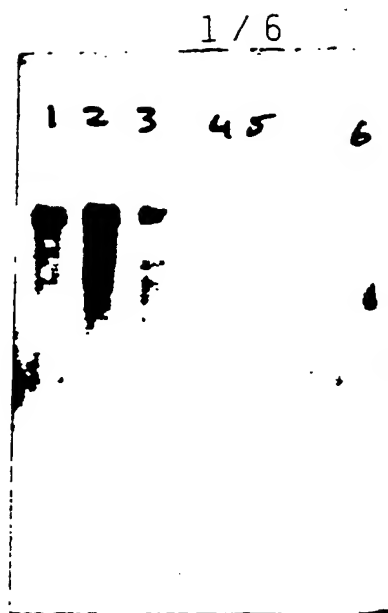
21. A genomic clone of Sh-2.

22. A genomic clone of Sh-2 as deposited with the ATCC under accession number 75129 and parts, variants and analogues thereof.

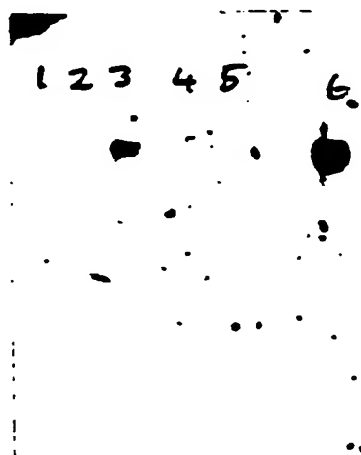
23. A genomic clone of Sh-2 having a DNA sequence substantially as set out in SEQ ID No: 1 of the Sequence Listing.

24. A genomic clone of Bt-2.

25. A genomic clone of Bt-2 as deposited with the ATCC under accession number 75130 and parts, analogues and variant thereof.



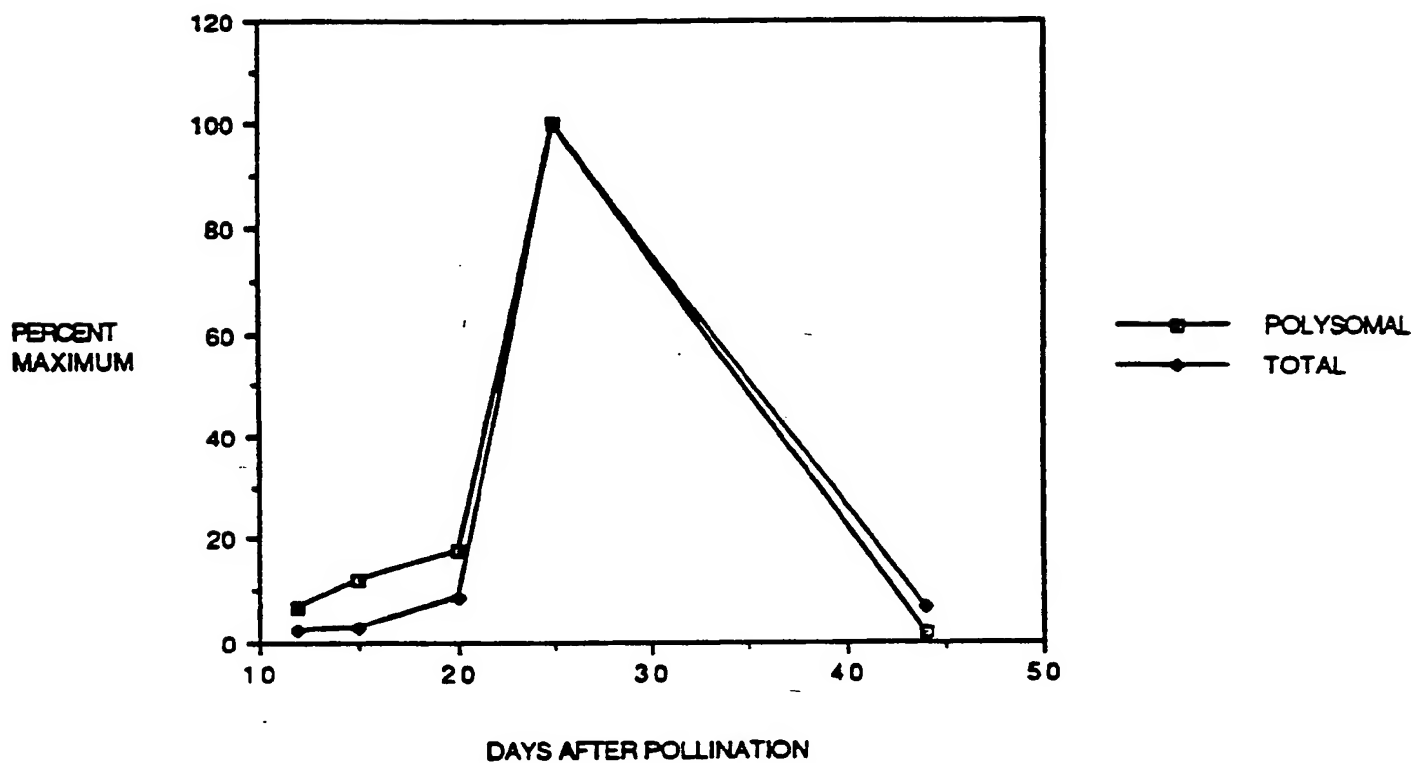
**Figure 1. Sh-2 mRNA in 201 X 202 and 101** Northern blot probed with <sup>32</sup>P-labelled Sh-2 cDNA. Lanes: 1-5 201 X 202; 1-2.5 ug 25 dpp endosperm bound polysomal RNA; 2-2.5 ug 25 dpp endosperm free polysomal RNA; 3-10 ug 25 dpp total endosperm RNA; 4-10 ug leaf RNA; 5-10 ug root RNA; 6-10 ug 21 dap 101 RNA.



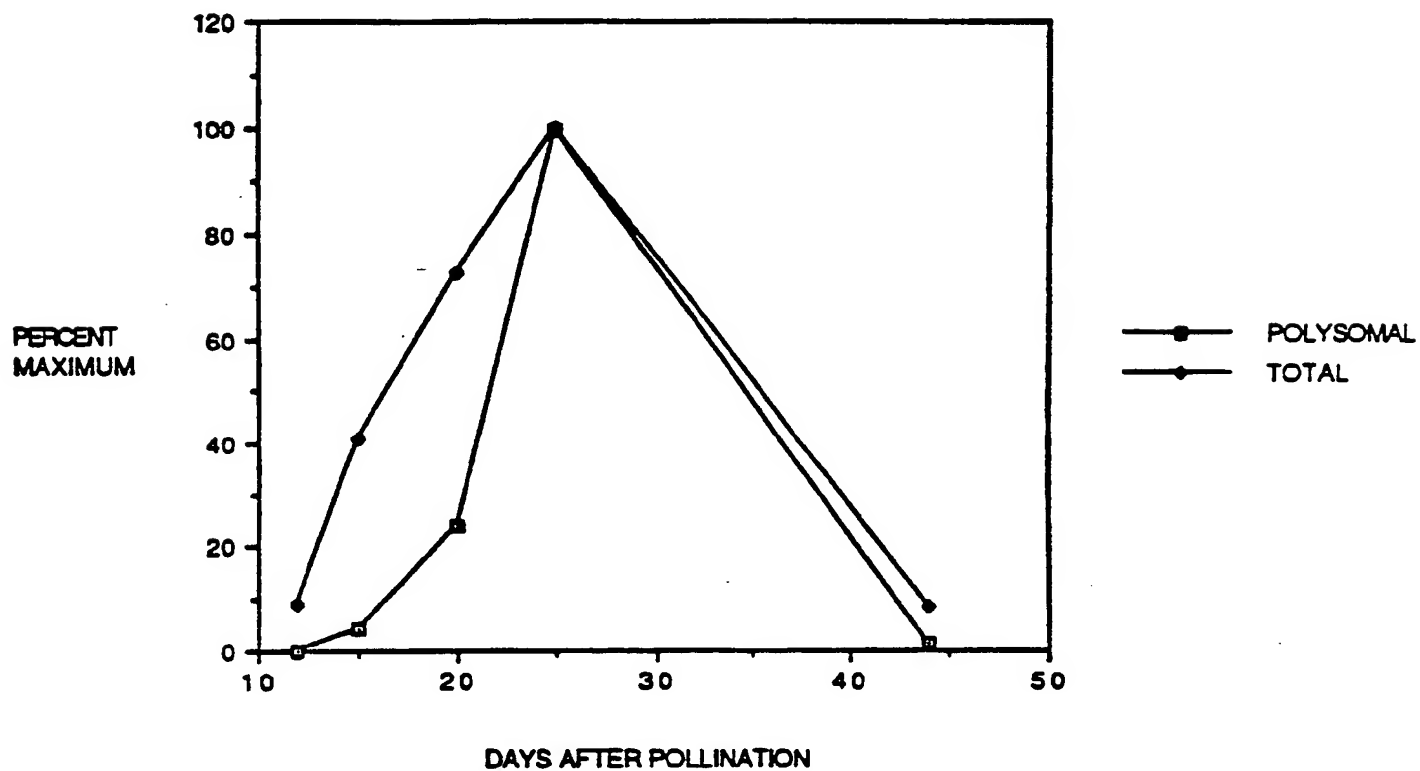
**Figure 2. Bt-2 mRNA in 201 X 202 and 101** The blot in Figure 1 was stripped and subsequently probed with <sup>32</sup>P-labelled Bt-2 cDNA.

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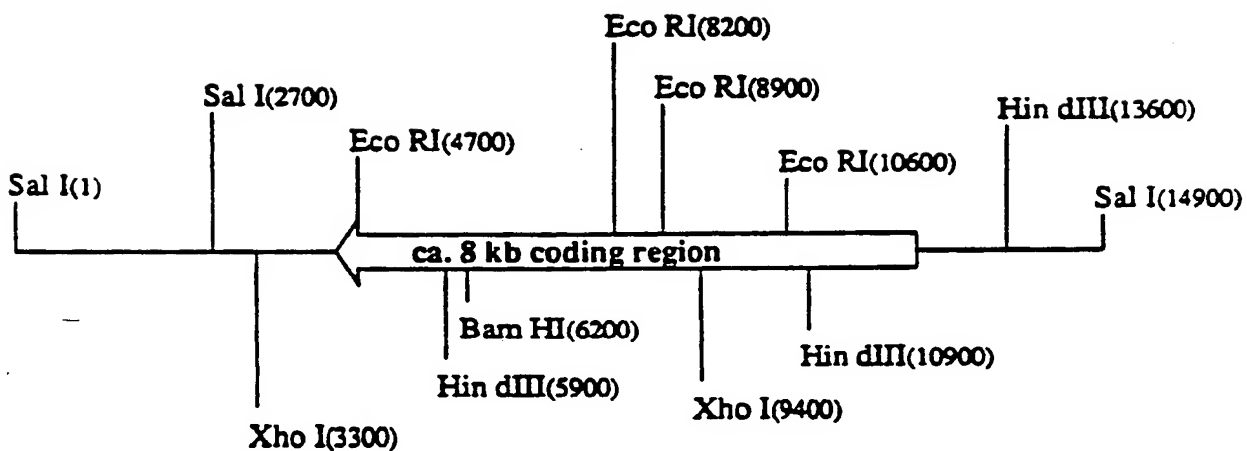
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**FIGURE 3. DEVELOPMENTAL PROFILE OF BT-2****SUBSTITUTE SHEET**

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**FIGURE 4. DEVELOPMENTAL PROFILE OF SH-2****SUBSTITUTE SHEET**

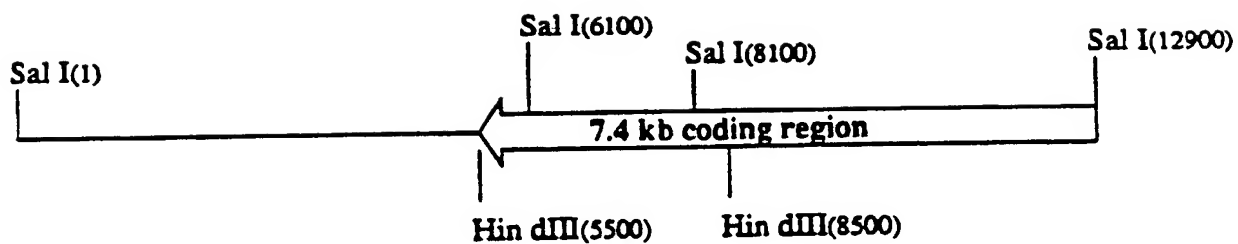
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**Figure 5. Composite Restriction Map of the *Sh-2* Genomic Clone.** Composite map of clones  $\lambda$  4.2 and  $\lambda$  8.2.2. Regions hybridizing to the *Sh-2* cDNA are indicated by the arrow.

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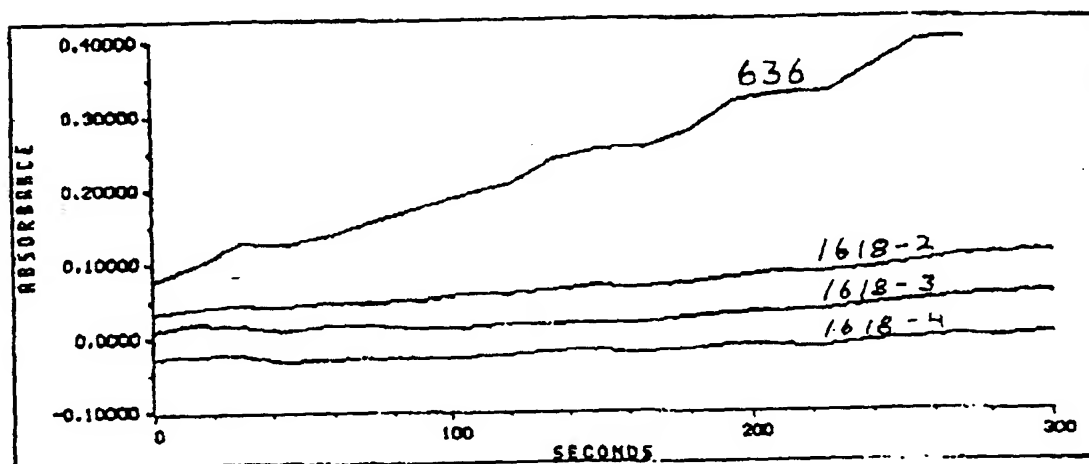
**Figure 6. Restriction Map of the *Bt-2* Genomic Clone.** The arrow indicates regions hybridizing to the *Bt-2* cDNA.

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Figure 1. ADP glucose pyrophosphorylase activity of anti-sense transformants.

17.5 ug of total soluble cellular protein was added to each assay. The assay is coupled to NADP reduction and absorbance at 340 nm is measured.



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## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 92/02531

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> <small>When several classification symbols apply, indicate all<sup>6</sup></small>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/82; C12N15/54; C12N15/11; A01H5/00		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; A01H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,9 000 856 (DNA PLANT TECHNOLOGY) 8 February 1990 see the whole document ---	1
Y	GENE vol. 97, no. 2, 15 January 1991, AMSTERDAM NL pages 199 - 205 ANDERSON, J.M., ET AL. 'Molecular characterization of the gene encoding a rice endosperm-specific ADPglucose pyrophosphorylase subunit and its developmental pattern of transcription' see the whole document --- -/--	21-25
<p><sup>10</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
22 FEBRUARY 1993		09. 03. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1983)

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>THE PLANT CELL. vol. 2, no. 6, June 1990, ROCKVILLE, MD, USA. pages 581 - 588 BHAVE, M.R., ET AL. 'Identification and molecular characterization of Shrunk-2 cDNA clones of maize' see the whole document</p>	21-23
Y	<p>PLANT PHYSIOLOGY. vol. 92, 1990, ROCKVILLE, MD, USA. pages 881 - 885 PREISS, J., ET AL. 'Molecular characterization of the Brittle-2 gene effect on maize endosperm ADPglucose pyrophosphorylase subunits' see the whole document</p>	24,25
P,X	<p>PLANT PHYSIOLOGY. vol. 98, 1992, ROCKVILLE, MD, USA. pages 1214 - 1216 SHAW, J.R., ET AL. 'Genomic nucleotide sequence of the wild-type Shrunk-2 allele of Zea mays'</p>	21-23
A	<p>EP,A,0 368 506 (ICI) 16 May 1990 cited in the application see the whole document</p>	1-25
A	<p>CHEMICAL ABSTRACTS, vol. 115, 1991, Columbus, Ohio, US; abstract no. 249560, SONNEWALD, U., ET AL. 'Expression vectors for manipulation of protein or carbohydrate content of potato tubers' see abstract &amp; DE,A,4 013 144 (IGFB) 24 October 1991</p>	1-25
A	<p>BIOTECHNOLOGY vol. 10, no. 1, 1992, NEW YORK US pages 40 - 43 FRALEY, R. 'Sustaining the food supply' see page 41, column 3, line 43 - line 51 &amp; ABSTRACT 714 THIRD INTERNATIONAL CONGRESS PLANT MOLECULAR BIOLOGY, TUSCON, ARIZONA, OCTOBER 6-12, 1991. STARK, D.M., ET AL. 'Increased starch and dry matter deposition in transgenic Russet Burbank potato tubers'</p>	1-25

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9202531  
SA 66269

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

22/02/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9000856	08-02-90	None	
EP-A-0368506	16-05-90	AU-A- 4430789 JP-A- 2273177	16-08-90 07-11-90

EPO FORM P007

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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